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Impact of solar ultraviolet-B radiation on the antioxidant defense system in soybean lines differing in flavonoid contents

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Abstract

Exposure to ultraviolet-B (UV-B) radiation can lead to oxidative damage in plants. However, plants possess a number of UV-protection mechanisms including screening of potentially damaging UV-B and increased production or activities of antioxidants. The balance or trade-off between these two mechanisms has rarely been studied and is poorly understood. Two isolines of soybean (*Glycine max* [L.] Merr.) Clark cultivar, the normal line with moderate levels of flavonoids and the magenta line with reduced flavonoids levels, were grown in the field with or without natural levels of UV-B. Leaflet blades of the first trifoliate leaf were harvested after 4–12 days of exposure to the experimental conditions for analysis of active oxygen species (AOS) and antioxidant levels. Solar UV-B radiation caused oxidative stress in both lines and altered AOS metabolism primarily by decreasing superoxide dismutase activity and increasing the activities of ascorbate peroxidase, catalase and glutathione reductase. This resulted in decreased ascorbic acid content and increased dehydroascorbate content. The magenta line had greater oxidative stress than the normal line in spite of its enhanced oxidative defense capacity as compared to the normal line, even under UV-B exclusion. These results indicate enhanced sensitivity in the magenta line, especially under UV-B exclusion that was likely due to the absence of flavonoid epidermal screening compounds and subsequent increased penetration of solar ultraviolet radiation into the leaf.

Keywords: Active oxygen species; Antioxidants; Flavonoids; *Glycine max*; Oxidative stress; Soybean; UV-B radiation

1. Introduction

Increases in chlorofluorocarbons in the atmosphere may deplete the earth's stratospheric ozone layer (Molina and Rowland, 1974), and a decrease in the ozone column has led to an increase in levels of ultraviolet-B (UV-B: 280–320 nm) radiation that reach the earth's surface (Gleason et al., 1993; Blumthaler and Amback, 1990). Many studies have indicated deleterious effects of UV-B such as reduced growth and photosynthesis (Ruhland et al., 2005; Germ et al., 2005). Some of the mechanisms that could lead to this damage are damages to DNA (Bray and West, 2005) or oxidative stress (Yannarelli et al., 2006a; Yang et al., 2005).

However, many plants are quite resistant to UV-B radiation and possess a number of UV protection mechanisms. One of the most important mechanisms is screening out UV-B radiation by accumulation of flavonoids or other UV-absorbing compounds in the leaf epidermis (Schmelzer et al., 1988; Robberecht and Caldwell, 1983). Other mechanisms that have received less attention than epidermal screening mechanisms are enzymatic and nonenzymatic antioxidative defense systems that may mitigate UV-induced damage that occurs due to the production of active oxygen species (AOS). The extent to which each of these possible defense mechanisms is operative or whether there is a trade-off between screening mechanisms and mitigation mechanisms such as antioxidants is poorly understood. For example, reductions in antioxidants in return for enhanced screening capacity could have indirect consequences such as a decline in frost hardiness noted in several arctic species (Taulavuori et al., 2005). The influence of other environmental factors on carbon and nitrogen limitations could also interact to alter the balance or consequences of a trade-off between these defense mechanisms. However, a more in depth understanding of the generation and scavenging of AOS is needed before this relationship can be fully understood.

The AOS potentially induced by UV-B radiation include not only free radicals such as superoxide (O_2^-) and hydroxyl radicals ($^{\bullet}OH$), but also hydrogen peroxide (H_2O_2) and singlet

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oxygen ($^1O_2$). These AOS can cause oxidative damage to membrane lipids, nucleic acids, and proteins (Foyer et al., 1994). To keep this damage to a minimum, plants possess enzymatic and nonenzymatic antioxidantive defense systems. Among the latter are glutathione (GSH), ascorbic acid (AsA), α-tocopherol, carotenoids, flavonoids, mannitol and hydroquinones (Larson, 1988). The enzymatic antioxidants include enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (POD; EC 1.11.1.7), ascorbate peroxidase (APX; EC 1.11.1.11), glutathione reductase (GR; EC 1.6.4.2), dehydroascorbate reductase (DHAR; EC 1.8.5.1), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and others. SOD rapidly converts O_2^- to H_2O_2 which can then be converted to water and oxygen by CAT (Noctor and Foyer, 1998). However, CAT is found predominantly in the peroxisome and has low substrate affinity. An alternative mode of H_2O_2 destruction is via peroxidase (APX) which is found throughout the cell (Jimenez et al., 1997). APX uses two molecules of AsA to reduce H_2O_2 to water, with the generation of two molecules of monodehydroascorbate (MDHA); MDHA can be reduced to AsA, catalyzed by MDHAR, and AsA can also be nonenzymatically regenerated from MDHA. Dehydroascorbate (DHA) is always produced during the rapid disproportionation of the MDHA radical and DHA is then reduced to AsA by the action of DHAR using GSH as the reducing substrate. This results in the generation of glutathione disulphide (GSSG), which is reduced to GSH by GR. The removal of H_2O_2 through this series of reactions is known as the ascorbate–glutathione cycle (Noctor and Foyer, 1998).

Understanding the mechanisms for removal of AOS is important for UV studies because increasing evidence suggests that AOS are involved in the damage caused by UV-B radiation. For example, UV-B radiation has been shown to increase AOS levels (Kalbina and Strid, 2006; Hideg et al., 2003) and lipid peroxidation (Yannarelli et al., 2006b; Yang et al., 2005) in plants. Although it is not known how plants irradiated with UV-B generate AOS, it is thought that NADPH oxidase may be involved in the generation of AOS (Rao et al., 1996). Other studies have shown that UV-B radiation may have an impact on the nonenzymatic antioxidants such as AsA (Shiu and Lee, 2005; Giordano et al., 2004; Galatro et al., 2001), GSH (Galatro et al., 2001; Kalbin et al., 1997), and α-tocopherol (DeLong and Steffen, 1998). Studies on the effects of UV-B on the enzymatic antioxidants at both the activity level (Agrawal and Rathore, 2007; Yannarelli et al., 2006b) and the mRNA level (Willekens et al., 1994) have yielded inconsistent results so it is not clear how uniform this response among plant species and how this may be modified by concurrent environmental conditions.

Most of the above studies were conducted indoor using a growth chamber or greenhouse in which plants were exposed to high UV-B radiation and relatively lower levels of ultraviolet-A (UV-A: 320–400 nm) and photosynthetically active radiation (PAR: 400–700 nm). Responses of plants in controlled conditions may differ from those in the field conditions, because of alterations in the natural spectral balance of UV-B, UV-A and PAR (Caldwell et al., 2003; Krizek, 2004) and the balance of damage and repair mechanisms. Very few studies have been conducted on the impacts of solar UV-B radiation on AOS metabolism under natural environmental conditions (Agrawal and Rathore, 2007; Mazza et al., 1999; Taulavuori et al., 1998). It is important to make an extensive investigation of the status of various antioxidants after exposure to natural levels of UV-B so as to assess their contributions to defense systems.

Soybean (*Glycine max* [L.] Merr.) genotypes exhibit a wide range of sensitivity to UV-B radiation, due in part to differences in flavonoid contents (Reed et al., 1992). In addition to epidermal screening of potentially damaging UV-B radiation, flavonoids may also contribute to UV protection by scavenging of AOS. However, the role that flavonoids play in protection against AOS damage has been studied only under artificial condition in *Arabidopsis* (Rao et al., 1996; Rao and Ormord, 1995; Landry et al., 1995). Isolines of soybean that lack flavonoids provide a valuable tool for evaluating the protective role of flavonoids (screening or antioxidant roles) compared to non-flavonoid enzymatic or antioxidant mitigation mechanisms. Therefore, the purpose of this UV-B exclusion study was to evaluate the effects of ambient levels of UV-B radiation on the AOS system in soybean and to test the hypothesis that the absence of flavonoids would lead to enhanced antioxidant capacity in the magenta line as the primary means of UV-protection in that line.

2. Materials and methods

2.1. Plant materials and experiment design

Two soybean isolines with different flavonoids content were used: the Clark normal line which produces moderate levels of flavonol glycoside level, and the Clark magenta line that has greatly reduced flavonol glycoside levels (Buzzell et al., 1977). Seeds of these soybeans were planted in pots in greenhouse at the University of Maryland and allowed to germinate for 3 days. Following this period the plants were moved to the USDA, Beltsville Southfarm where they were separated into two UV-B treatment regimes. Half of the plants were placed inside either of two open-ended exclusion shelters made of polyester, which absorbs almost all solar radiation below 316 nm. The second half of plants was placed under another two shelters covered by clear Teflon material, which is virtually transparent to solar UV radiation. The materials are similar in transmission properties in the UV-A and PAR wavebands. The plants beneath the polyester filters received very little UV-B radiation and served as controls for seasonal changes in temperature and PAR, etc. Plants were rotated every day and watered to minimize the occurrence of drought stress which can alter the response to UV-B radiation (Sullivan and Teramura, 1990). Plants were harvested three times at 4-day intervals following the production of the first trifoliate leafs. The leaflet blades of the first trifoliate from three to five plants from each replicate treatment were harvested for each sample and five independent samples were harvested for each treatment replicate. The harvested samples were frozen in liquid nitrogen immediately and then stored at −80 °C prior to analysis.

2.2. Determination of enzyme activities

Plant tissues were ground in mortar with liquid nitrogen, and ice-cold extraction buffer (100 mM KH_2PO_4/K_2HPO_4, pH 7.8; 1% Triton X-100; 5 mM AsA; 1% PVP) were added. After 15 min at 4 °C, the homogenates were centrifuged at 21,000 × *g* for 10 min. The supernatants were passed through a Sephadex G-25 column (PD-10) which had been equilibrated with 100 mM KH_2PO_4/K_2HPO_4 buffer, pH 7.8 containing 5 mM AsA. The elutants were collected for enzymes activities. All assays were performed at 25 °C, had a final volume of 1 mL and were performed in triplicates. Protein concentrations were determined according to Bradford method (1976) with bovine serum albumin (BSA) as a standard.

APX activity was measured by the method of Nakano and Asada (1981). The reaction mixture contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 0.25 mM AsA, 0.2 mM H_2O_2 and extract. The APX activity was determined by following the decrease in A_{290}, extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to calculate activity. Correction was made for the low, nonenzymatic oxidation of AsA by H_2O_2. MDHAR activity was measured by the method of Hossain et al. (1984) at 340 nm. Reaction mixture contained 50 mM Tris–HCl (pH 7.6), 0.2 mM NADH, 2.5 mM AsA, 1 unit of ascorbate oxidase and extract. Extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate activity. To determine the MDHAR activity the rate of MDHA-independent NADH oxidation (without AsA and AsA oxidase) was subtracted from the MDHA-dependent oxidation rate (with AsA and AsA oxidase). DHAR activity was measured as described by Nakano and Asada (1981) at 265 nm. The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 2.5 mM GSH, 0.2 mM DHA, 0.1 mM EDTA and extract. Extinction coefficient of 14 mM^{-1} cm^{-1} was used to calculate activity. The reaction was corrected for nonenzymatic reaction of DHA by GSH. GR activity was measured by the method of Foyer and Halliwell (1976) at 340 nm. The reaction mixture contained 50 mM HEPES–KOH (pH 8.0), 1.0 mM EDTA, 0.2 mM NADPH, 1.0 mM GSSG and extract. Reaction was started by the addition of GSSG. Extinction coefficient of 6.22 mM^{-1} cm^{-1} was used to calculate activity. Correction was made for the small, nonenzymatic oxidation of NADPH by GSSG. CAT activity was determined as H_2O_2 consumption measured as the decrease in absorbance at 240 nm according to the method of Aebi (1983). The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.0), 10 mM H_2O_2 in phosphate buffer. Extinction coefficient of 39.4 mM^{-1} cm^{-1} was used to calculate activity. SOD activity was measured spectrophotometrically by measuring the inhibition of O^{2-} dependent reduction of cytochrome *c* at 550 nm, according to the method of Tanaka and Suigahara (1980). The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 7.8), 0.1 mM EDTA, 0.1 mM Cyt *c*, 0.1 mM xanthine, enzyme extract and xanthine oxidase. One unit of SOD was defined as the quantity of enzyme required to inhibit the reduction of Cyt *c* by 50%. Guaiacol POD activity was measured at 436 nm by the method of Nakano and Asada (1981). The assay contained 50 mM KH_2PO_4/K_2HPO_4 (pH 5.25), 40 mM guaiacol, 10 mM H_2O_2, and the extract. The activity was calculated using an extinction coefficient of 25.5 mM^{-1} cm^{-1}.

2.3. Determination of lipid peroxidation, AsA, glutathione and H_2O_2 contents

Samples were ground with mortar and pestle at 0 °C for 10 min in the presence of 5% trichloroacetic acid (TCA). The slurry was centrifuged at 16,000 × *g* for 5 min at 4 °C. The supernatant was used to determine the content of thiobarbituric acid reacting substances (TBARS) or neutralized to pH 6 (for ASA) or 7 (for GSH and H_2O_2) with 4N KOH. All assays were performed in triplicates.

The lipid peroxidation was determined by the TBARS contents according to the method of Dhindsa and Matowe (1981). The supernatant was combined with equal volume of thiobarbituric acid (TBA) reagent (0.5% TBA in 20% TCA), heated at 95 °C for 30 min, cooled and centrifuged. The amount of TBARS in the supernatant was determined from the difference between the absorbance at 532 nm and that at 600 nm using extinction coefficient of 155 mM^{-1} cm^{-1}.

H_2O_2 was determined according to the method of Okuda et al. (1991), 200 μL of the supernatant was applied to 1-mL column of anion exchange resin (AG-1, Bio-Rad), and the column was washed with 800 μL of distilled water, the eluate was used for H_2O_2 assay. The reaction mixture contained 1 mL of the elute, 400 μL of 12.5 mM 3-dimethylaminobenzic acid in 375 mM phosphate buffer (pH 6.5), 80 μL of 10 mM 3-methyl-2-benzothiazoline hydrazone and 20 μL of peroxidase (0.25 unit). After 3 min at 20 °C the absorbency was determined at 590 nm and compared with increases elicited by standard samples of hydrogen peroxide.

AsA and DHA were determined by a method of Foyer et al. (1983). AsA was measured by the change in A_{265} following the addition of ascorbate oxidase. Sample was added to 0.1 mM phosphate buffer (pH 5.6) to make a final volume of 990 μL. The A_{265} was measured and 10 μL (4 units) of ascorbate oxidase was added. The AsA content was measured via the absorption decrease using an extinction coefficient of 14 mM^{-1} cm^{-1}. DHA was measured by the same method following reduction to AsA in a reaction mixture containing 20 mM dithiothreitol and 50 mM phosphate buffer (pH 7.0).

Glutathione was determined according to the procedure of Anderson et al. (1992). Total glutathione was measured in reaction mixture consisting of 400 μL reagent A containing 110 mM Na_2HPO_4, 40 mM NaH_2PO_4, 15 mM EDTA, 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and 0.04% BSA; 320 μL reagent B containing 1 mM EDTA, 50 mM imidazole, 0.02% BSA and an equivalent of 1.5 units GR activity; 400 μL of 1:50 dilution of the extract in 5% Na_2HPO_4 (pH 7.5) prepared immediately prior to starting the assay; and 80 μL of 9 mM NADPH to start the reaction. Change in absorbance was measured at 412 nm. For GSSG, 1 mL of the 1:50 dilution was incubated for 1 h at 25 °C with 40 μL of 2-vinylpyridine. The standard curve in which the GSH equivalents present was plotted against the rate of change in A_{412}. For each sample, GSH was estimated as the difference between total glutathione and GSSG.

2.4. Statistical analysis

The experimental design was a split plot with the main plots being UV-B treatment and replicated twice and the subplots being the soybean lines. The General Linear Model procedure (SAS PC version 6.04, SAS Institute, Inc., Cary, NC) was used for the different analysis of variance (ANOVA) to test for main effects of UV-B, lines, time and their interactions.

3. Results

3.1. TBARS and H_2O_2 contents

The levels of TBARS and all metabolites measured varied with sampling time (Table 1). Levels of TBARS also were increased by ambient solar UV-B radiation ($P = 0.0002$; Table 1 and Fig. 1A) at 4 and 8 days. However, the differences in TBARS contents did not differ between the two soybean lines ($P = 0.0733$), although the magenta line had higher TBARS content than the normal line at 4 days ($P = 0.0001$). Ambient UV-B radiation had no impact on the leaf H_2O_2 content ($P = 0.2177$) but the magenta line had higher H_2O_2 content than the normal line at 4 and 8 days ($P < 0.0001$; Table 1 and Fig. 1B).

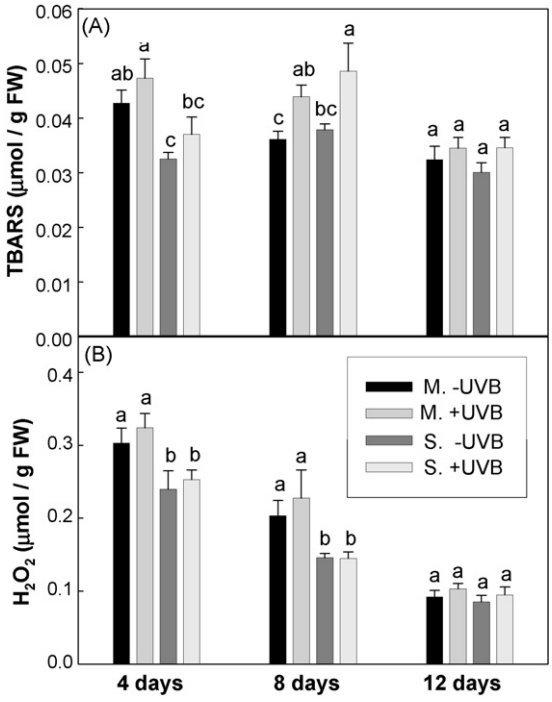

Fig. 1. The influence of UV-B radiation on contents of TBARS (A) and H_2O_2 (B) in the Clark normal line and the magenta line of soybean grown in the field under either ambient or reduced levels of solar UV-B radiation. Each bar is the mean ± S.E. ($n = 10$) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

3.2. AsA and glutathione contents

AsA and glutathione contents were determined to examine the effect of ambient UV-B on the water-soluble antioxidants. The AsA content was decreased by UV-B ($P = 0.0002$) while DHA and total AsA content (AsA + DHA) were significantly increased by UV-B ($P < 0.0001$ and $P = 0.0069$, respectively; Table 1 and Fig. 2). Therefore, the ratio of AsA/DHA was decreased after exposure to UV-B radiation ($P < 0.0001$). The magenta line had a higher content of DHA and total AsA than the normal line ($P = 0.0208$ and $P = 0.0484$, respectively; Table 1 and Fig. 2). There were no differences between these two lines in the contents of AsA or the ratio of AsA/DHA.

The GSH and total glutathione contents were not affected by UV-B but the GSSG contents were decreased by solar UV-B radiation ($P = 0.0377$; Table 1 and Fig. 3). Therefore, the ratio of GSH/GSSG was enhanced after UV-B exposure ($P = 0.027$). The

Table 1
P table for the effects of UV-B (U), line (L), time (*T*) and their interactions on the metabolite contents and enzyme activities of soybean plants grown in the field under either ambient or reduced levels of UV-B radiation

Parameter	U	L	*T*	U*L	U**T*	*T*L	U**T*L
TBARS	0.0002	0.0733	<.0001	0.5516	.2331	0.0014	0.9070
H_2O_2	0.2177	<.0001	<.0001	0.5918	0.9661	0.0271	0.8965
AsA	0.0002	0.8909	<.0001	0.5182	0.0985	0.8132	0.0099
DHA	<.0001	0.0208	<.0001	0.3065	0.1962	0.0023	0.2948
AsA/DHA	<.0001	0.4845	<.0001	0.6645	0.0022	0.2008	0.0169
AsA + DHA	0.0069	0.0484	<.0001	0.5719	0.1585	0.0073	0.3312
GSH	0.3752	0.0369	<.0001	0.3934	0.9224	0.0026	0.7323
GSSG	0.0377	0.8289	<.0001	0.8111	0.0986	0.9299	0.5379
GSH/GSSG	0.0270	0.1774	<.0001	0.6138	0.1065	0.0185	0.2781
GSH + GSSG	0.5735	0.0420	<.0001	0.3940	0.9817	0.0038	0.7843
SOD	<.0001	<.0001	<.0001	0.0250	0.0491	0.0001	0.9380
CAT	0.0067	<.0001	0.0121	0.3502	0.6862	<.0001	0.8528
POD	0.8323	0.5109	<.0001	0.9776	0.8504	0.8953	0.8286
GR	0.0203	<.0001	<.0001	0.4823	0.5599	0.0141	0.4752
DHAR	0.2507	<.0001	<.0001	0.6384	0.6059	0.4816	0.7500
MDHAR	0.1124	<.0001	<.0001	0.2124	0.8547	0.1186	0.7571
APX	<.0001	0.0088	<.0001	0.0984	0.0757	0.1231	0.3655

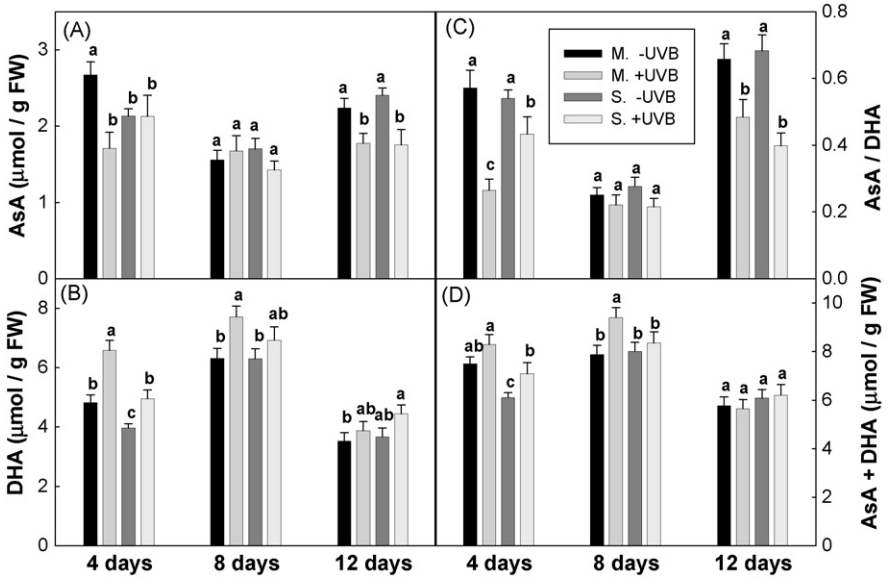

Fig. 2. The influence of UV-B radiation on contents of the AsA pools in the Clark normal line and the magenta line of soybean grown in the field under either ambient or reduced levels of solar UV-B radiation. Each bar is the mean ± S.E. ($n = 10$) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

magenta line had higher levels of GSH and total glutathione than the normal line ($P = 0.0369$ and 0.042, respectively). However, there were no differences between these two lines in the GSSG content and the ratio of GSH/GSSG.

3.3. Enzyme activities

Solar UV-B radiation decreased the SOD activity ($P < 0.0001$; Table 1 and Fig. 4A) and increased the CAT activity ($P = 0.0067$) at 8 days in the magenta line only (Fig. 4B). However, SOD activity was greater in the normal Clark line compared to the magenta line. Guaiacol POD activity greatly increased at 12 days but was not affected by ambient solar UV-B radiation during the experiment and there was no difference in its activity between the two lines (Table 1 and Fig. 4C).

The magenta line also had higher activities of MDHAR, DHAR, and GR, than the normal line (all at $P < 0.0001$; Table 1 and Fig. 5). Unlike SOD activity which was reduced in the magenta compared to the normal soybean line, APX activity was greater in the magenta than the normal line (Table 1 and Fig. 5). The activity of GR and APX was also increased by solar UV-B exposure ($P = 0.0067$ and < 0.0001, respectively) while the activity of MDHAR and DHAR were not affected by solar UV-B radiation. The activity of GR and APX was also greater

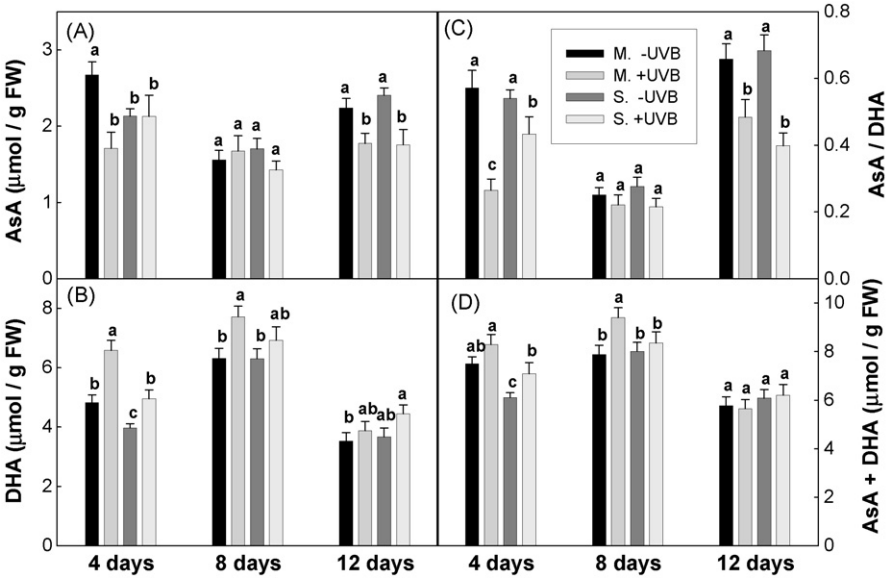

Fig. 3. The influence of UV-B radiation on contents of glutathione pools in the Clark normal line and the magenta line of soybean grown in the field under either ambient or reduced levels of solar UV-B radiation. Each bar is the mean ± S.E. ($n = 10$) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

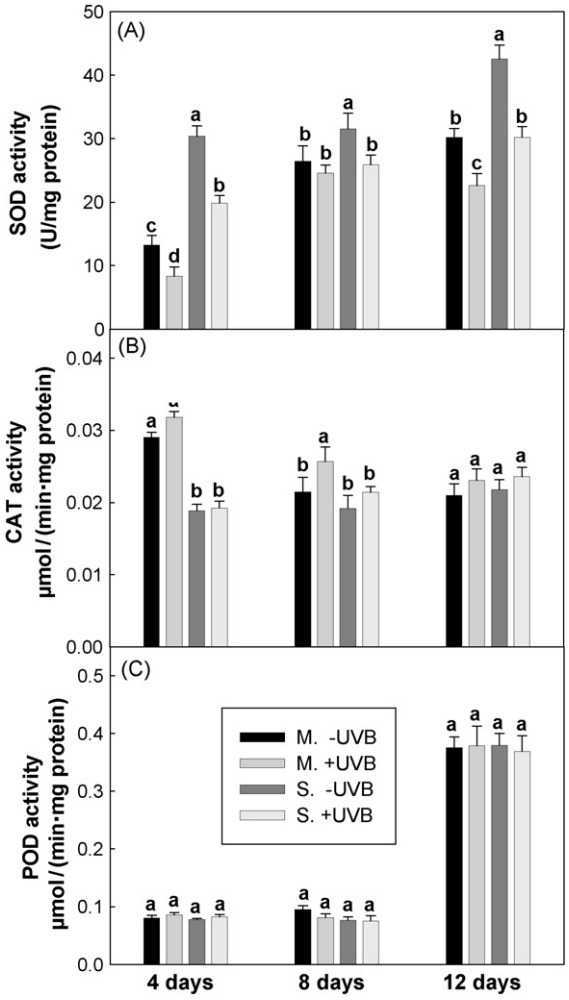

Fig. 4. The influence of UV-B radiation on activities of SOD (A), CAT (B), and POD (C) in the Clark normal line and the magenta line of soybean. Each bar is the mean ± S.E. ($n = 10$) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

in the magenta than the normal soybean lines ($P < 0.0001$ and $P = 0.0088$, respectively, Table 1).

4. Discussion

Previous studies on the effects of UV-B on lipid peroxidation have shown that under low or moderate levels of UV-B, no increased lipid peroxidation was detected (Giordano et al., 2004; Dai et al., 1997). However, high levels of UV-B were shown to increase peroxidation (Yannarelli et al., 2006b; Hideg et al., 2003; Costa et al., 2002; Alexieva et al., 2001). All these experiments were conducted in growth chamber or greenhouse under artificial conditions. In field supplementation studies, UV-B increased lipid peroxidation in buckthorn (Yang et al., 2005), but its effect varied among soybean cultivars (Yanqun et al., 2003). The increased TBARS content under ambient levels of solar UV-B found in this study suggests that oxidative damage may occur in some species under ambient levels of UV-B radiation and this was more apparent in the magenta line.

It should be noted however, that the TBARS method has been shown to over estimate lipid peroxidation under some conditions (Hodges et al., 1999). However, Taulavuori et al. (2001) also found that when measurements were made during the active growth period, as was done in this study, there was minimal difference between the method used here and the corrected method proposed by Hodges et al. (1999). Also, the emphasis in this study was the determination of relative changes in peroxidation levels rather than on absolute quantitation of lipid peroxidation so the consequences of an over estimation of peroxidation levels, as long as it was a systematic over estimate, would not likely alter the comparative results in this study.

Hydrogen peroxide is known to diffuse across biological membranes and cause cellular damage. In this experiment, solar UV-B did not affect H_2O_2 content, which might be due to the increased APX activity and decreased SOD activity. Therefore, this increased lipid peroxidation was likely caused by AOS other than H_2O_2. Although the O_2^- content was not determined in this study, the inhibition of SOD activity by UV-B could lead to increases in O_2^- content. However, this does not necessarily mean that UV-B has no effect on H_2O_2 generation. UV-B exposure enhanced NADPH-oxidase in *Arabidopsis* (Rao et al., 1996), and the involvement of NADPH-oxidase in H_2O_2 generation has been demonstrated in some plants (Rao et al., 1996; Moller and Lim, 1986). H_2O_2 production was increased by high levels of UV-B in several studies conducted indoors (Kalbina and Strid, 2006; Hideg et al., 2003; Alexieva et al., 2001). However, Murphy (1990) could not detect H_2O_2 accumulation in cultured rose cells treated with broadband radiation including UV-B. There is very limited information about the effects of UV-B on H_2O_2 content in higher plants under field conditions so it is unclear whether a general response to ambient levels of UV-B is increased H_2O_2 accumulation.

Changes in several antioxidant pools in response to UV-B suggest an induction of these damage mitigation mechanisms in response to AOS generation. For example, AsA, a major primary antioxidant reacting directly with hydroxyl radicals, superoxide and singlet oxygen, and also a powerful secondary antioxidant reducing the oxidized form of α-tocopherol, was highlighted in a study using AsA-deficient *Arabidopsis* mutant (Conklin et al., 1996). Increases in the AsA pool in response to UV-B exposure have also been observed in several species (Galatro et al., 2001; Dai et al., 1997; Takeuchi et al., 1996; Rao and Ormord, 1995). However, in maize seedling, UV-B exposure had no effect on the AsA content (Carletti et al., 2003). Under field conditions, long-term exposure to enhanced levels of UV-B did not change the AsA content in *Vaccinnium myrtillus* (Taulavuori et al., 1998) although they reported trends for a reduction in AsA by UV-B exposure. In this study, the AsA content was decreased but the DHA content and total AsA + DHA pool was increased under ambient UV-B radiation, resulting in a decreased ratio of AsA/DHA. These UV-B effects on AsA are consistent with the results in wheat and bean (Agrawal and Rathore, 2007) and this could be explained by the increase of APX activity under UV-B exposure. Higher APX activity consumes more AsA and produces more DHA.

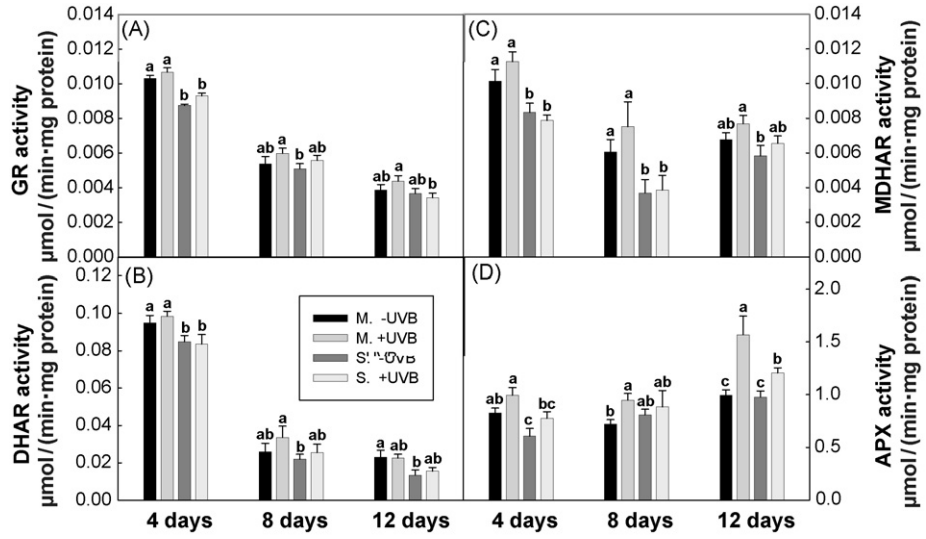

Fig. 5. The influence of UV-B radiation on activities of GR (A), DHAR (B), MDHAR (C), and APX (D) in the Clark normal line and the magenta line of soybean grown in the field under either ambient or reduced levels of solar UV-B radiation. Each bar is the mean ± S.E. ($n = 10$) for each treatment. Bars with the same letter were not significantly different at $\alpha < 0.05$.

The glutathione pool was also slightly affected by solar UV-B exposure in this study where only GSSG content was decreased by UV-B radiation. Increased thiol content by UV-B radiation has been reported in several studies (Galatro et al., 2001; Kalbin et al., 1997; Dai et al., 1997; Rao and Ormord, 1995), but all these increases were found under high levels of UV-B. In the field, long-term exposure to enhanced UV-B radiation did not affect thiol content in *V. myrtillus* (Taulavuori et al., 1998), but increased it in wheat and bean (Agrawal and Rathore, 2007).

Like the antioxidant pools, the activities of several key enzymes involved in AOS metabolism were altered by UV radiation. Contrasting responses of SOD to UV-B exposure have been reported revealing no uniform responses. For example, SOD activity was increased by UV-B radiation in pea and wheat (Alexieva et al., 2001), *Arabidopsis* (Rao and Ormord, 1995), *Lemna gibba* (Babu et al., 2003) and rice (Dai et al., 1997), but was not affected in barley (Mazza et al., 1999) and soybean (Malanga et al., 1999), and was decreased in sunflower cotyledon (Costa et al., 2002). Also, SOD expression was not affected by UV-B radiation in *Nicotiana plumbaginifolia* L. (Willekens et al., 1994), but was decreased in *Pisum sativum* (Strid, 1993). In a field study, supplemental UV-B increased SOD activity in wheat and bean (Agrawal and Rathore, 2007), and caused different responses among soybean cultivars (Yanqun et al., 2003). In other experiments the effects of artificial UV-B on SOD activity were found to vary with temperature (Takeuchi et al., 1996), duration of the treatment (Dai et al., 1997), leaf age and PAR source even under the same level of PAR (Krizek et al., 1993). Takeuchi et al. (1996) reported that UV-B increased SOD activity at 20 °C and decreased it at 25 °C in cucumber cotyledons. This would be consistent with the results of this study which was conducted during periods of high temperature in July. Rao et al. (1996) demonstrated that UV-B exposure preferentially induces peroxidase-related enzymes instead of SOD and the results of this study are consistent with that study.

In contrast to SOD, in this study UV-B radiation increased the APX activity. This is consistent with the results of several other studies conducted in chambers (Yannarelli et al., 2006b; Takeuchi et al., 1996; Rao et al., 1996; Landry et al., 1995) or in the field (Mazza et al., 1999) and suggests that APX has an important role in the control of endogenous H_2O_2 content. Solar UV-B radiation also increased the activities of CAT and GR, but had no impact on the activities of POD, MDHAR, and DHAR. It is also possible that measures of only total activities of enzymes may not adequately reflect UV-induced compartment-specific changes or enzyme alterations that do not change total activity. For example, UV-B could differently regulate enzyme isoforms as reported for POD (Murali et al., 1988), CAT (Willekens et al., 1994), SOD (Babu et al., 2003; Rao et al., 1996) and APX (Yannarelli et al., 2006a) in previous studies. More studies are needed to resolve these issues.

Overall the magenta line was more responsive in these parameters than the normal line and this would be consistent with a hypothesis that the antioxidant system could be up-regulated in the absence of an effective pool of screening compounds. The magenta line had higher H_2O_2 content than the normal line. Also the magenta line had higher contents of DHA, GSH, and total glutathione than the normal Clark soybean line, as well as higher activities of APX, DHAR, MDHAR, GR, and CAT, and lower SOD activity. These results suggest that oxidative stress and damage was greater in the magenta than in the normal line, in spite of its enhanced oxidative defense capacity as compared to the normal line and this occurred even in the absence of UV-B radiation. The enhanced responses in the magenta line, especially under UV-B exclusion, may be due to increased penetration of solar ultraviolet-A and/or oxidative stress caused by other stressors. However, in field studies (Sullivan et al., unpublished data) we have not seen evidence of severe physiological damage or reduced growth in these lines, even in the presence of increased levels of DNA damage (Pope, 2000). Also, even without flavonoids *per se*, epidermal-screening capacity in the

magenta line does increase in an exposure specific manner, particularly to levels of UV-A radiation (Sullivan et al., 2007). The combination of hydroxycinnamates found in the magenta line, and increased antioxidant capacity observed in this study appears to protect these plants from severe UV-B damage in the field.

The apparent oxidative stress and differences between the soybean lines grown under UV-B exclusion points to the role of UV-A in AOS metabolism. Although, it is less damaging on per photon basic than UV-B, UV-A comprises a much larger portion of the solar spectrum than does UV-B and UV-A is able to penetrate to greater depths within the leaf than UV-B (Liakoura et al., 2003). UV-A can cause photoinhibition of PSII (Krause et al., 1999), oxidative damage (Taulavuori et al., 2005; Yao et al., 2006) and growth inhibition (Krizek and Chalker-Scott, 2005; Flint and Caldwell, 2003) in higher plants. It can also induce oxidative stress and lipid oxidation in cyanobacteria (He et al., 2002) and alga (White and Jahnke, 2002) and was found to contribute 50% or more of UV damage in Antarctic phytoplankton (Cullen et al., 1992). Therefore, UV-A is an important photobiological component of sunlight and these data suggest that it plays a role in oxidative stress in soybean.

While flavonoids provide UV-B protection these data also infer a protective role of flavonoids against UV-A as well. Flavonoids accumulation in the epidermis would limit UV-A as well as UV-B penetration into mesophyll. The greater responses in the magenta line, even under UV-B exclusion, could be due to their inability to synthesize flavonoids and thus transmit increased fluxes of UV-A through their more transparent epidermis. Other studies indicated that *Arabidopsis* mutants lacking phenolic sunscreens exhibited enhanced responses in AOS metabolism upon UV-B exposure, but there was no difference under UV-B exclusion (Rao et al., 1996; Landry et al., 1995; Rao and Ormord, 1995). This points to subtle differences in experimental protocols, namely exclusion versus supplementation and to our lack of a basic understanding of the kinetics (e.g. linear, threshold or saturation responses) of the response to UV-B radiation. Further studies are needed to understand these responses and the impacts of solar UV-B, UV-A, and PAR irradiance on the AOS metabolism.

5. Conclusion

Our results indicate that solar UV-B caused oxidative stress in both lines, and altered the antioxidant defenses mainly by decreasing SOD activity and increasing activities of APX, CAT, and GR. The magenta line that does not produce flavonoids exhibited greater oxidative stress and damage than the normal line even though it demonstrated enhanced oxidative defense capacity as compared to the normal line. Since this was the case even under UV-B exclusion, the enhanced responses in the magenta line, suggests that the increased penetration of UV-A, in the absence of flavonoids in the epidermis may be physiologically important and warrants further investigation. Finally, it suggests that antioxidants other than flavonoids may respond to UV-B independent of flavonoids and provides protection in their absence. The balance or interactions between these two UV protection mechanisms, screening and damage mitigation, needs further investigation.

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